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Prologue: In support of Award number W81XWH-10-1-0430, we are pleased to present the following annual report detailing progress made towards the completion of work described in the Statement of Work associated with this award.

Introduction

The purpose of the research supported by this award is to determine if targeting the hedgehog signaling pathway in breast cancer can reduce breast cancer recurrence. Two specific facts about breast cancer recurrence highlight the need for better treatment modalities that target and prevent disease recurrence.

1. In the United States and other countries with access to advanced cancer care, local and distant breast cancer recurrence accounts for ~95% of breast cancer mortality(Jemal et al., 2010).
2. The life-time risk of breast cancer recurrence among survivors is greater than the life-time risk of developing breast cancer in the general population(Jemal et al., 2010).

Together these two statements suggest that while substantial progress has been made in treating primary breast cancer, those treatments do not efficiently reduce the risk of disease recurrence. Therefore there is a need for novel treatment strategies. Disease recurrence is believed to be the result of a subset of tumor cells with two distinct features; broad spectrum resistance to therapeutics and tumorigenicity(Hurt and Farrar, 2008; Pardal et al., 2003; Polyak and Weinberg, 2009; Woodward et al., 2005). Our previous studies identified a regulatory relationship between ΔNp63α a protein that is required for long-term preservation of epithelial stem cells(Mills et al., 1999; Yang et al., 1999) and the hedgehog-signaling pathway that governed stem cell quiescence(Li et al., 2008). Stem cell quiescence is necessary to preserve long-term replicative capacity while simultaneously avoiding the detrimental effects of excessive proliferation. It is also a potent blockade to cellular differentiation(Coller et al., 2006). Based upon this we put forth the hypothesis that the hedgehog signaling pathway could be targeted to subvert quiescence in stem cell populations. Doing so would force these cells back into the cell cycle, thereby sensitizing them to adjuvant cancer therapeutics. To test this hypothesis we developed three specific tasks that are described in detail in the Statement of Work. Since this award was a collaborative IDEA award we sought to take advantage of the hedgehog-signaling expertise of Dr. David J. Robbins and the mammary stem cell and breast cancer expertise of Dr. James DiRenzo. What follows is a detailed description of progress towards the completion of the research supported by this award.

Body

Statement of Work

Work to be completed by the Robbins Lab
Work to be completed by the DiRenzo Lab
Work that will be completed in both Labs

Task 1: Determine the effects of pharmacologic regulation of Smoothened on mammary stem cell quiescence.

Sub-Task 1: Evaluate the effects of Smo agonists on regulation of quiescence in Lin^- /$CD24^+$/$CD29^{high}$ cells.

A: Establish a treatment model based on one utilized according to the Frank-Kamenetsky model.

1: Isolate and quantify $Lin^-/CD24^+/CD29^{high}$ and $Lin^-/CD24^+/CD29^{low}$ to determine if the ratio of mammary stem cells to committed mammary progenitors is altered by Smo activation.

2: Evaluate mammosphere initiating capacity of $Lin^-/CD24^+/CD29^{high}$ -/+ Smo agonist.

3: Determine the effects of Smo agonists on engraftment efficiency, ductal elongation and side-branching.

Sub-Task 2: Evaluate the ability of Smo antagonists to rescue the $Ptch1^{-/+}$ phenotype.

A: Identical treatment model.

1: Isolate and quantify $Lin^-/CD24^+/CD29^{high}$ and $Lin^-/CD24^+/CD29^{low}$ to determine if the ratio of mammary stem cells to committed mammary progenitors is altered by Smo antagonism.

2: Evaluate mammosphere initiating capacity of $Lin^-/CD24^+/CD29^{high}$ from $Ptch1^{-/+}$ mice -/+ Smo antagonist.

3: Determine the effects of Smo agonists on engraftment efficiency, ductal elongation and sidebranching.

Outcome 1: Results of these studies will determine if pharmacologic regulation of hedgehog signaling can alter the state of quiescence versus activation in mammary stem cells. The studies of Frank-Kementsky have established a treatment model in which the Smoothened agonist was sufficient to partially rescue the Sonic Hedgehog knockout suggesting that similar treatment strategies will enable use to evaluate the in vivo effects of these compounds on mammary stem cell quiescence.

Outcome 2: Completion of these studies will also provide important information regarding the pharmacology of Smoothened antagonists in the mammary gland.

Outcome 3: Completion of these studies will also enable us to determine the reversibility of hedgehog-mediated activation of mammary stem cells. We have previously shown that hedgehog activation via Ptch1 heterozygosity results in a quiescence defect. Studies described in Sub-task 2 will enable us to determine if this phenotype can be rescued by inhibition of Smoothened such a finding would provide evidence of the reversibility of this signal.

our previous finding that hedgehog signaling acts in a mitogenic manner on mammary stem cells to subvert their quiescence.

Task 2: Determine if hedgehog-mediated defective quiescence predisposes mulliparous MMTV-myc mice to tumorigenesis.

Sub-Task 1: Genetic analysis: Cross FV/b MMTV-myc with B6/129 $Ptch1^{-/+}$

A: 25% $Ptch1^{+/+}$ + No MMTV-myc Tg (WT on Mixed FV/bxB6/129 bkg)

B: 25% $Ptch1^{-/+}$ + No MMTV-myc Tg (Ptch on Mixed FV/bxB6/129 bkg)

C: 25% $Ptch1^{+/+}$ + MMTV-myc Tg (MMTV-myc on Mixed FV/bxB6/129 bkg)

D: 25% $Ptch1^{-/+}$ + MMTV-myc Tg ($Ptch1^{-/+}$ + MMTV-myc Mixed FV/bxB6/129 bkg)

1: Collect 8 females from each genotype and monitor for tumorigenicity over 12 months.

Sub-Task 2: "Pseudo-genetic analysis"

A: Isolate $Lin^-/CD24^+/CD29^{high}$ from MMTV-myc and infect with pGIPZ-non-specific shRNA or pGIPZ-mPtch1 shRNA

B: Sort GFP-ve from GFP+ve.

C: Transplant GFP+ve cells into cleared fat pads of 3 week old FV/B recipients

1: Transplant 16 glands (i.e. 8 recipient mice) with $Lin^-/CD24^+/CD29^{high}$ infected with pGIPZ-non-specific shRNA.

a: Monitor transplants for tumorigenesis over 12 months.

2: Transplant 16 glands from 8 mice with $Lin^-/CD24^+/CD29^{high}$ infected with with pGIPZ-mPtch1 shRNA.

a: Monitor transplants for tumorigenesis over 12 months.

Outcome 1: While the intent of the proposal is to determine if hedgehog-mediated activation of stem cells disrupts quiescence and sensitizes the cells to cancer therapeutics this study represents a unique opportunity to determine if defective quiescence is a predisposing factor for breast cancer. Under normal conditions, nulliparous MMTV-myc have a very low incidence of tumorigenesis and parity increases the incidence to ~ 100% within 12 months of a complete pregnancy. We hypothesize that the forfeiture of mammary stem cell quiescence that occurs during pregnancy-associated mammary gland expansion potentiates myc-mediated carcinogenesis. Both the pure genetic and "pseudo-genetic" studies described here will enable us to determine if ectopic c-myc on a background of defective quiescence obviates the need for parity in this model. Such a finding would implicate control of quiescence in breast cancer initiation and would further suggest that mammary stem cells that are actively dividing and express an oncogenic allele contribute to tumor initiation. It would also underscore the rationale for driving these cells into the cell cycle as a means to sensitize them to cancer treatment.

Task 3: Evaluate the ability of hedgehog activation to sensitize tumor stem cells to taxanes in vitro and in vivo.

Sub-Task 1: In vitro studies

A: Isolate $Lin^-/CD24^+/CD29^{high}$ from wt and $Ptch1^{-/+}$ mice and evaluate the effects of paclitaxel on mammosphere formation.

B: Isolate $Lin^-/CD24^+/CD29^{high}$ from wt mice and evaluate the ability of a Smoothened agonists to sensitize cells to paclitaxel.

1: Isolation of $Lin^-/CD24^+/CD29^{high}$ from wt mice

2: Pre-treatment with paclitaxel.

3: Culture under low-binding conditions that favor expansion of self-renewing populations.

C: Isolate $Lin^-/CD24^+/CD29^{high}$ from $Ptch1^{-/+}$ mice and evaluate the ability of Smo antagonist to protect cells from paclitaxel.

Sub-Task 2: In vivo studies

A: Determine the efficiency of serial transplantation of tumors derived from MMTV-myc and $Ptch1^{-/+}$ x MMTV-myc following paclitaxel treatment.

1: Cross FV/b MMTV-myc with B6/129 $Ptch1^{-/+}$

a: 25% $Ptch1^{+/+}$ + No MMTV-myc Tg

b: 25% $Ptch1^{-/+}$ + No MMTV-myc Tg

c: 25% Ptch1$^{+/+}$ + MMTV-myc Tg
d: 25% Ptch1$^{-/+}$ + MMTV-myc Tg

B: Collect 8 females from each genotype and impregnate them.
1: This is necessary to increase the incidence of tumorigenesis to ~100%.
2: Expectation is that tumors will arise in the MMTV-myc and Ptch1$^{-/+}$ X MMTV-myc.

C: Monitor for tumorigenesis

D: When tumors reach 1 cm in diameter begin treating with paclitaxel or vehicle control.
1: Use the established paclitaxel treatment model.

E: Monitor tumor regression and stop treatment when tumors are < 0.5cm.

F: Isolate Lin^{-}/CD44^{+}/CD24$^{low/-}$ cells from remaining tumors
1: Monitor activation of apoptotic programs in these cells.
a: Gene expression analysis via Q-PCR
b: In situ TUNEL staining
2: Transplant into syngeneic wt mice
3: Monitor tumor initiation.

Outcome 1: Completion of this experiment will determine if disruption in stem cell quiescence in the setting of breast cancer (as opposed to normal mammary gland stasis) will sensitize these cells to paclitaxel treatment. Such a finding could be translated into clinical studies that are designed to selectively activate tumor stem cells in the context of adjuvant chemotherapy or radiation treatment. The intermediate goal of the PI is to direct such a trial in conjuction with clinicians associated with the Comprenhensive Breast Program at the Norris Cotton Cancer Center, which is directed by the PI.

Reportable results for Annual Report

Efforts to complete work associated with Task 1 of the Statement of Work associated with this proposal have lead to the establishment of an in vivo treatment model based upon the one described by Frank-Kamenetsky et. al(Frank-Kamenetsky et al., 2002). Multiple technical difficulties with the development and use of Smoothened agonists have delayed completion of studies described in Sub-task A.3. Our anticipated results have not been observed to date however several controls that are essential for the quality control of the in vivo pharmacology of the drug have been sub-optimal suggesting that compounds with improved pharmacokinetics may be necessary to proceed with this aim. In contrast to these difficulties studies that aimed to reduce or inhibit aberrant side-branching of lobulo-alveolar formation in the Ptch1-/+ mice have demonstrated that inhibition of Smoothened with cyclopamine, but not SANT1 diminished side-branching and lobulo-alveolar development. This latter result indicates that inhibiton of HH signaling can be achieved pharmacologically with cyclopamine. To avoid known toxicities associated with cyclopamine, studies were limited to 14 days of treatment which was sufficient to blunt side-branching and alveolar formation. We believe that this result is consistent with the hypothesis that hedgehog signaling is promoting mammary regenerative kinetics. This is consistent with
Task1: Determine the effects of pharmacologic regulation of Smoothened on mammary stem cell quiescence.

Sub-task 1: Evaluate the effects of Smo agonists on regulation of quiescence in Lin-/CD24+/CD29high cells.

Sub-task 2: Evaluate the ability of Smo antagonists to rescue the Ptch1-/+ phenotype.

We began this work by establishing our mouse colonies and growing them up to an appropriate size. The mice used in this project are the Ptch1$^{tm1Mps/J}$ from The Jackson Laboratory (Stock#003081). A sufficient number of breeders were established in order to obtain an adequate number of females of both genotypes in each litter. We next began to work out the sorting procedures that will be used in subsequent experiments. We isolated MECs from wt and Ptch+/- mice by generating a single cell suspension from freshly dissected mammary glands.

Quantification of mammary stem cells (Lin-/CD24+/CD29high) and mammary progenitors (Lin-/CD24+/CD29low), which were separated by FACS analysis, was then done. The ratio of mammary stem cells to mammary progenitors was then compared in both groups of animals. Results show a dramatic increase in the ratio of luminal progenitors to mammary stem cells in the Ptch1+/- when compare to wt mice. This result suggests increased commitment of the stem cell population to luminal progenitors when HH pathway is activated.

We are currently working in refining the FACS analysis and increasing the number of subjects to confirm those results. The ability to establish a robust protocol for isolating both mammary epithelial cell populations is central to successfully complete the objectives presented in SOW. To validate the differences observed in commitment of stem or progenitor cells in the presence of an abnormal activation of Hedgehog pathway we performed a colony formation assay, comparing the capacity of mammary stem cell from wt mice and Ptch1+/- to give rise to

luminal, myoepithelial or mixed colonies. MECs were isolated and cultured under low attachment conditions and the resulting mammospheres disaggregated and plated at clonogenic density for 8 days, then stained for luminal and myoepithelial markers. The number of colonies was compared from both groups of animals. A reduction of an 80% in the relative number of mixed colonies in the Ptch+/- cells compare to wt was observed. The decrease in the number of myoepithelial cells in the heterozygous mice is also in accordance with the role of HH pathway on stem cell linage commitment. We are now performing the same results but culturing the Ptch1+/- cells in the presence of cyclopamine to rescue the reduction of myoepithelial cells. The result of the colony formation assay is a functional readout for changes in the stem cell population that will complement the FACS analysis, which depends on various cellular markers. This assay is useful to further evaluate the effects of pharmacologic regulation of Smoothened on mammary stem cell quiescence- **Task 1 in the SOW**.

We have also begun our analyses of the dose/response treatment in Ptch1+/- mice using a HH antagonist- **Task 1 in the SOW**. A single dose was administered by oral gavage, and the mammary glands harvested 48h later. The ratio of mammary stem cells to mammary progenitors was then compared to that from vehicle control treated mice. We are in the process of completing these experiments, as well as treating wt mice with a hedgehog agonist to optimize the dose and duration of such treatments for subsequent experiments. Finding the drug, dose, vehicle and method of administration that efficiently modify hedgehog signaling in the mammary epithelial cells of treated mice will be necessary in order to evaluate the effect of those alterations in the quiescence capacity of the mammary stem cells, and further its taxane sensitivity- **Task 2 in the SOW**.

Efforts towards the completion of **Task 2** are ongoing. Presently we have developed a genetic crossing strategy and genotyping method for the development and identification of mice that are MMTV-myc$^{+/-}$/Ptch1$^{+/+}$ or MMTV-myc$^{+/-}$/Ptch1$^{+/LacZ}$. At the time of this writing we have developed 12 mice that are MMTV-myc$^{+/-}$/Ptch1$^{+/+}$ and 15 that are MMTV-myc$^{+/-}$/Ptch1$^{+/LacZ}$. These mice have been housed with identical genotype littermates and are monitored twice weekly for tumorigenesis. Presently no additional tumorigenesis has been noted in either genotype, however we have noted a significant increase in size and body mass among the MMTV-myc$^{+/-}$/Ptch1$^{+/LacZ}$ which is poorly understood at this time.

Task 2 also aims to determine the effects of ectopic c-myc in wild type and Ptch1-/+ mammary stem cells. Lin^{-}/CD24^{+}/CD29^{high} fractions that are enriched for mammary stem cells have been isolated from Ptch1-/+ mice and wild-type littermates and cultured them on irradiated NIH 3T3 cells for 5 days in the presence of a retrovirus programmed to over-express c-myc. Initial efforts lead to disappointing viarl infection efficiency and we are in the process of developing a similar virus that expresses green fluorescent protein (GFP) so that in future studies infected cells can be sorted from uninfected cells. Doing so will enable a direct side-by-side comparison of engraftment efficiency, ductal outgrowth and tumorigenesis.

Task 3 aimed to investigate whether pharmacologic modulators of hedgehog signaling are able to sensitize quiescent cells such as mammary stem cells to cytotoxic chemotherapeutics such as taxanes. These studies are largely pending due to technical difficulties associated with Task 1. We have repeated our previously reported result showing that mammary stem cells

from Ptch-/+ mice have a quiescence defect and are developing culture conditions for these cells that will be amenable to the question of taxane sensitivity. Doing so will circumvent the technical difficulties with the pharmacologic hedgehog modulators.

Key Research Accomplishments

1. Demonstration of dramatically enhanced luminal epithelial commitment in reponse to constituative hedgehog signaling in the Ptch1-/+ mouse.

2. Demonstration of enhanced formation of luminal mammospheres at the expense of bi-potent spheres by mammary stem cells from Ptch1-/+ mice relative to those from wild-type littermates.

3. Demonstration of a proliferative advantage, or quiescence defect in mammary stem cells in which hedgehog signaling is persistent.

4. Development of cohorts of MMTV-myc$^{+/-}$/Ptch1$^{+/+}$ and MMTV-myc$^{+/-}$/Ptch1$^{+/LacZ}$ for the determination of whether defective mammary stem cell quiescence is condition of breast cancer predisposition.

5. In vitro expansion of enriched fractions of mammary stem cells (Lin^{-}/CD24^{+}/CD29^{high}) by culturing on irradiated NIH3T3 cells.

6. Achieved ectopic expression of c-myc in mammary stem cells from wild-type and Ptch1-/+ mice via retroviral transduction.

Reportable outcomes

1. Cell fate analysis from in vitro expansion of wild-type and Ptch-/+ mammary stem cells have indicated that persistent hedgehog signaling via Ptch1 heterozygosity preferentially promotes luminal cell fate specification. This was observed by an increase in the ratio of luminal progenitors to stem cells in Ptch1-/+ mice relative to wild-type mice. IT was confirmed by analysis of colony outgrowths from enriched fractions of mammary stem cells from wt and Ptch1-/+ mice, which showed that the ration of luminal colonies to bipotent colonies was significantly elevated in Ptch-/+ colonies relative to wt colonies. We believe this result is consistent with the observation that Ptch-/+ mammary stem cells are defective in quiescence. These results are also consistent with the hypothesis that aberrant hedgehog signaling results in both stem cell proliferation and luminal commitment that can be best explained bi asymmetric mitosis.

2. We have noted a highly significant increase in size and body mass in mice that are MMTV-myc$^{+/-}$/Ptch1$^{+/LacZ}$ relative to mice that are MMTV-myc$^{+/-}$/Ptch1$^{+/+}$. Currently these mice are being monitored for tumorigenesis, however we intend to evaluate the causes of the increased size and body mass.

3. We have been able to achieve successful gene-transfer in cultured mammary stem cells and are improving the efficiency of this process for planned transplantation experiments.

Conclusions

1. At this mid-point of the funding period there are only limited conclusions that can be drawn. Clearly we can conclude that hedgehog signaling results alteration of cell fate from bipotent to luminal cell fates. In addition to the information that this finding gives us regarding the role of hedgehog signaling in the mammary regenerative hierarchy, it also suggests that this assay may be adapted to in vitro and in vivo pharmacology experiments that will address questions raised in Task1 and will enable completion of Task 3.

2. A second conclusion that must be acknowledged is the unanticipated difficulties of using pharmacologic inhibitors of hedgehog signaling in vivo. We have redoubled our efforts to establish and perfect a treatment model that will be adaptable to longer term tumorigenesis studies as well as to the toxicity studies in Task 3.

References

Coller, H.A., Sang, L., and Roberts, J.M. (2006). A new description of cellular quiescence. PLoS Biol *4*, e83.

Frank-Kamenetsky, M., Zhang, X.M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F.Y., Jones, S., Shulok, J.*, et al.* (2002). Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. Journal of biology *1*, 10.

Hurt, E.M., and Farrar, W.L. (2008). Cancer stem cells: the seeds of metastasis? Mol Interv *8*, 140-142.

Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010). Cancer statistics, 2010. CA Cancer J Clin *60*, 277-300.

Li, N., Singh, S., Cherukuri, P., Li, H., Yuan, Z., Ellisen, L.W., Wang, B., Robbins, D., and DiRenzo, J. (2008). Reciprocal intraepithelial interactions between TP63 and hedgehog signaling regulate quiescence and activation of progenitor elaboration by mammary stem cells. Stem Cells *26*, 1253-1264.

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature *398*, 708-713.

Pardal, R., Clarke, M.F., and Morrison, S.J. (2003). Applying the principles of stem-cell biology to cancer. Nat Rev Cancer *3*, 895-902.

Polyak, K., and Weinberg, R.A. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer *9*, 265-273.

Woodward, W.A., Chen, M.S., Behbod, F., and Rosen, J.M. (2005). On mammary stem cells. J Cell Sci *118*, 3585-3594.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C.*, et al.* (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature *398*, 714-718.